Stability of limonin glucoside in beverage matrices

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Abstract

BACKGROUND: We completed a study over a 200-day period examining the stability of limonin glucoside formulated into three beverage matrices.

RESULTS: Beverages containing limonin glucoside were found to contain limonin (0.13–20.10 mg L^{-1}) during their initial testing; however, these concentrations were directly attributable to the presence of contaminating limonin in the particular lot of limonin glucoside used to prepare the beverage and did not increase over the test period. Likewise, limonin glucoside concentrations did not vary significantly, with the exception of the beverage matrix that included vitamin B_2. Exposure of the vitamin B_2-containing beverages to light resulted in a rapid reduction in the limonin glucoside content. Liquid chromatographic–mass spectrometric and nuclear magnetic resonance results from the analyses of pre- and post-light exposed beverages suggest photooxidation of the furan moiety as the likely degradation pathway.

CONCLUSION: Results from this study indicate that limonin glucoside is resistant to degradation into limonin, the stability of limonin glucoside formulated into beverages exceeds six months and that limonin glucoside should not be formulated into beverages containing vitamin B_2 unless the beverages are protected from light.
Published in 2008 by John Wiley & Sons, Ltd.

Keywords: *Citrus*; limonoids; beverage; limonin glucoside

INTRODUCTION

Citrus fruits and juices are one of the most healthful components of the human diet and much of the positive contribution to human health, nutrition, and consumer acceptance may be attributed to the presence of plant secondary metabolites in fruits and juices. Included amongst the most abundant secondary metabolites are citrus limonoids, a family of highly oxygenated triterpenoid compounds. These secondary metabolites have been screened for biological activities, and antitumor, anti-HIV and potentially cholesterol-lowering properties[1] are among the reported benefits for these compounds. In addition, the human bioavailability of limonin glucoside has been reported.[2]

Citrus limonoids are found in a variety of citrus tissues as aglycones, glucosides or A-ring lactones, the metabolic precursors to limonoid aglycones and glucosides (Fig. 1). Limonin and limonin glucoside (LG) are the most abundant aglycone and glucoside for most citrus species.[3] Limonin glucoside is water soluble and tasteless. In contrast, limonin is bitter and due to poor water solubility ($<40\,mg\,L^{-1}$) is typically found at concentrations of $<20\,mg\,L^{-1}$ in juices.[4,5] The gradual accumulation of limonin in citrus fruits or juices following freeze damage or processing is the predominant cause for the development of *delayed bitterness* in citrus products. Limonin concentrations as low as 6 mg L^{-1} render citrus products unacceptable to consumers.[6,7]

Since little is known about the stability of LG in matrices other than juice, we undertook a study to examine its stability in three beverage matrices similar to those used for sports drinks. These beverages were placed under various storage conditions and assessed for their limonin and LG content for more than 200 days. As a margin of safety, a self-imposed maximum of 3 mg L^{-1} limonin was set for the beverages to be considered acceptable. Reported here are the results for the stability studies.

EXPERIMENTAL

Materials and chemicals

Solvents (acetonitrile, chloroform, methanol) were high-performance liquid chromatographic (HPLC) grade and along with perchloric acid (70%, ACS reagent) glacial acetic acid (ACS Plus), and formic acid (88%, ACS reagent grade) were purchased from Fisher Scientific Ltd (Waltham, MA, USA).

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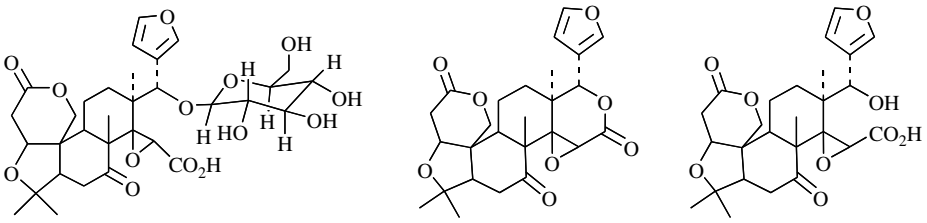
(*Received 25 January 2008; accepted 16 June 2008*)
DOI: 10.1002/jsfa.3344

 J Sci Food Agric 0022–5142/2008/$30.00



Limonin glucoside
(Limonin 17 β-D-glucopyranoside, LG)

Limonin

Limonoate A-ring lactone

Figure 1. Structures of limonin glucoside (1), limonin (2), and limonoate A-ring lactone.

Deuterated methanol (98.8 + %D), dimethyl 3,4-furandicarboxylate, and 4-(dimethylamino)benzaldehyde (DMAB) were obtained from Sigma Aldrich (St Louis, MO, USA). Water was deionized to $\geq$18.1 MΩ cm^{-1} resistance using a Barnstead nanopure deionization system (Dubuque, IA, USA) and filtered through a 0.45 μm type HA membrane filter (Millipore, Billerica, MA, USA) prior to use. Strata-X solid-phase extraction (SPE) columns (30 mg/1 mL and 200 mg/3 mL) were purchased from Phenomenex (Torrance, CA, USA). Pure crystalline limonin and LG were available as analytical standards in our laboratory.

Preparation of beverages

Beverages were prepared using LG isolated by our laboratory and packaged into transparent plastic containers. Three groups of test beverages were formulated based on a 12 oz (354 mL) serving. All test beverages were prepared in water and for each group a different production lot of LG was used. Group 1 consisted of three beverages targeted at 0, 0.500 and 2.000 g dose levels of LG prepared in a beverage matrix that included citrate, vitamin C, and flavoring and coloring agents. Group 2 consisted of three beverages; two were targeted at 0 and 0.250 g dose levels of LG prepared in a beverage matrix that included citrate, vitamins C and B2, and flavoring and coloring agents; the third beverage contained only citrate and LG (0.250 g). Beverages for Group 3 consisted of two LG dose levels at 0 and 0.287 g and were prepared in a matrix that included citrate, vitamins C and B_2, and flavoring and coloring agents.

Sampling of beverages

Beverages from Groups 1 and 2 were stored in a food refrigerator and periodically sampled for their limonin and LG content over a 200-day period. Group 3 beverages, in addition to being stored in a food refrigerator, were also stored at room temperature on a bench top and under refrigeration in a glass-door chromatography refrigerator in order to simulate conditions found in a retail environment. Sampling of Group 3 beverages was terminated after 3 weeks (see Results and discussion section for more details).

Extraction of limonin from beverages

An aliquot of beverage (1.0 mL) was combined with 2.0 mL of chloroform and vortexed for 1 min at speed #4 on a VWR multi-tube vortexer (West Chester, PA, USA). The resulting emulsion was clarified by centrifugation for 2 min at 2800 rpm using a Fisher Scientific Marathon 8K centrifuge (Waltham, MA, USA). Half of the chloroform layer (1.0 mL) was transferred to a 1.2 mL polypropylene tube and evaporated to dryness under a stream of warm nitrogen gas (Jones Chromatography SPE Dry Dual, Charlottesville, VA, USA). Prior to HPLC analysis, samples were reconstituted in a solution (500 μL) comprising 30% acetonitrile and 70% H_2O, vortexed for 1 min at speed #4 and the sample pooled to the bottom of its tube by centrifugation for 2 min at 4000 rpm (ICE Centra CL4, Needham Heights, MA, USA). Samples were extracted in triplicate.

HPLC analysis for quantification of limonin

Limonin concentrations present in the chloroform extracts of the samples were determined by HPLC using a Waters 2695 System (Milford, MA, USA) coupled to a Waters 996 PDA detector set to scan from 190 to 250 nm or Waters dual absorbance detector 2487 set at 210 nm. Standards and samples (20 μL) were injected on a 50 × 2.0 mm Phenomenex Phenosphere-Next-5 μ phenyl column equipped with a guard column of the same stationary phase, maintained at 30 °C and flowing isocratically at a rate of 0.5 mL min^{-1}. The solvent composition was 30:70 acetonitrile–10 mmol L^{-1} formic acid and the total run time was 5.5 min. Instrument control and data acquisition were accomplished using Masslynx (Version 4.0). Quantitative results were obtained by comparing the area under the curve at 210 nm for the sample to a standard curve generated from limonin (1.0, 2.5, 5.0, 10.0, and 25.0 mg L^{-1}) standards prepared in 20% acetonitrile. Values were reported as the mean of three replicates.

Estimation of total limonoid glucoside content

The limonoid glucoside content of each sample was estimated by the colorimetric method of Breksa and Ibarra[5] with the following modification. In place of SPE treatment, samples were diluted 10:1 or 20:1 with water and acetonitrile to a final acetonitrile

content of 30% and the diluted solutions analyzed directly. Samples with concentrations in excess of the calibration range were further diluted with 30% acetonitrile (aq) and reassessed. For spike recovery experiments, diluted samples in 30% acetonitrile (aq) were combined 1:1 with an LG solution (100 mg L^{-1}, 30% acetonitrile). Samples were analyzed in triplicate.

Preparation of samples for analysis by HPLC–electrospray ionization/mass spectrometry (ESI/MS) and nuclear magnetic resonance (NMR)

Prior to HPLC-ESI/MS and NMR analyses, samples were treated by SPE. For those samples destined for HPLC-ESI/MS analysis, a 0.5 mL aliquot of either a 10:1 or 20:1 dilution of the beverage in water was applied to a Strata-X SPE column (30 mg/1 mL) that had been washed with MeOH (2 mL) and equilibrated in water (2 mL). The flow-through and a subsequent water wash (2 mL) were discarded. After drying under vacuum for 1 min the column was eluted with MeOH (2 mL). The resulting eluent was collected and evaporated to dryness. Samples were reconstituted in 20% acetonitrile (0.5 mL) prior to analysis.

In preparation of analysis by NMR, undiluted samples (3 mL) were applied to Strata-X SPE columns (200 mg/3 mL) that had been washed with MeOH (2 mL) and equilibrated in water (2 mL). The flow-through and a subsequent water wash (2 mL) were discarded. After drying under vacuum for 1 min the column was eluted with MeOH (2 mL). The extraction was repeated twice and the collected eluents for each individual beverage combined prior to evaporation.

HPLC-ESI/MS analysis

The HPLC-ESI/MS analysis of samples for the presence of LG was accomplished using a system controlled by Masslynx (Version 4.1) and consisted of a Waters 600 controller and pump, Gilson 215 Liquid Handler (Middleton, WI, USA), and a Rainin model CH-1 column heater (Oakland, CA, USA) coupled to a Waters 996 PDA detector (190–250 nm scan range) and Waters ZQ 4000 mass spectrometer. Standards and samples (10 µL) were injected on a 50 × 2.0 mm Phenomenex Phenosphere-Next-5 µ phenyl column equipped with a guard column of the same stationary phase, maintained at 30 °C and flowing isocratically at a rate of 1.0 mL min^{-1}. The solvent composition was 85% of 4.0 mmol L^{-1} formic acid, 15% acetonitrile, and the total run time was 4.0 min. The flow from the Waters 996 detector was directed through an LC Packing's Acurate (Sunnyvale, CA, USA) flow splitter, such that only 1/4 of the flow was introduced into the mass spectrometer. Tuning of the mass spectrometer was accomplished in negative ion mode through optimization on the LG signal at *m/z* 649.3 generated by introduction of an LG solution (5 mg L^{-1}) into the mass spectrometer in the LC mobile phase at the flow rate used for analysis. The mass spectrometer was operated in the negative ion mode, with a desolvation temperature of 200 °C, capillary voltage of 3.0 kV, and cone voltage of 60 V. The mass spectrometer was set to acquire data over a mass range from 400 to 950 *m/z*.

NMR analysis

Samples were reconstituted in CD_3OD (0.5 mL) that contained dimethyl 3,4-furan dicarboxylate (3 mg mL^{-1}) and TMS as internal standards. The same solution was used to prepare a set of LG standards (0.62, 1.25, 2.50, 5.00, 10.00 mg) to accompany the samples. Proton NMR spectra were obtained at 300 K on a Brüker model ARX400 spectrometer, equipped with a 5 mm broadband, multi-nuclei, triple-axis gradient probe, at a frequency of 400.13 MHz and a 90° pulse at a 7–8 s repetition rate. The integrated areas of the α-furan peaks of LG (7.36 and 7.65 ppm) were normalized against the integrated area of the furan protons of dimethyl 3,4-furan dicarboxylate (2H, 8.13 ppm). For simplicity the area of the furan protons of dimethyl 3,4-furan dicarboxylate was arbitrarily set to 200. Normalized values were used to calculate the concentration of LG present in the sample. Some samples were exposed to a high-intensity visible light source from a microscope and spectra acquired again.

RESULTS AND DISCUSSION

In addition to evaluating the stability of LG, some of the concerns we sought to resolve through this study included measuring the initial limonin concentrations found in the beverages, ascertaining to what extent the concentrations would change during storage and if they would surpass a self-imposed limit of 3 mg L^{-1}. The 3 mg L^{-1} limit was set to provide a margin of safety from the 6 mg L^{-1} bitterness threshold. Limonin concentration was determined by HPLC analysis of chloroform extracts of the beverages. The limit of quantification for this method is less than 0.10 mg L^{-1} and limonin recoveries typically range from 95% to 104%. Limonin concentrations found in the beverages from Groups 1 and 2 on their initial receipt and over the course of the study are displayed in Fig. 2. Each of the beverages was found to contain limonin and the concentrations found in the 0.500 and 2.000 g LG dose beverages from Group 1 (Fig. 2A) exceeded both the 3 mg L^{-1} self-imposed and 6 mg L^{-1} bitterness thresholds, whereas beverages from Group 2 (Fig. 2B) contained limonin concentrations less than 1 mg L^{-1}. The average limonin concentrations (±SD) for the beverages calculated using results from the entire study were 5.20 ± 0.85 and 20.07 ± 2.51 mg L^{-1} for the 0.500 and 2.000 g dose beverages in Group 1, respectively, and 0.31 ± 0.28 and 0.18 ± 0.11 mg L^{-1} for the 0.250 g and 0.250 g in citrate beverages, respectively. Over the course of the study, the limonin concentrations remained essentially unchanged.

Considering that all the beverages supplemented with LG contained limonin, there were two potential

DOI: 10.1002/jsfa

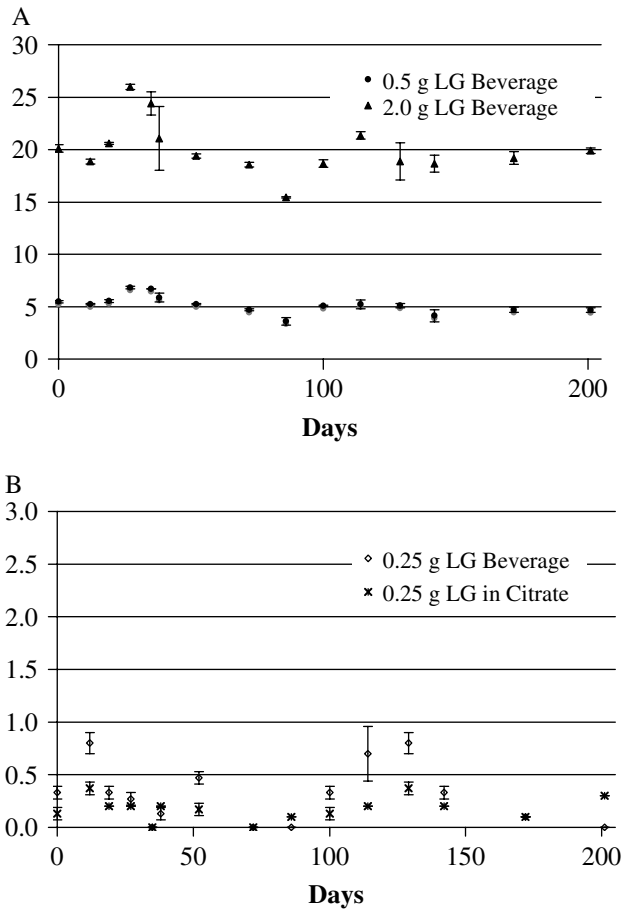


Figure 2. Limonin concentrations results for the beverages prepared at 0.500 g and 2.000 g per dose LG from Group 1 (A) and 0.250 g per dose LG beverages, matrix and citrate only, from Group 2 (B) are displayed. Beverages were extracted with chloroform and the limonin concentrations in the extracts determined by HPLC. Displayed values are from the average of three replicate analyses ± SD.

explanations for the limonin concentrations initially observed: limonin was (1) generated during the preparation of the beverages or (2) present in the LG material used to prepare the beverages. However, the first hypothesis is less probable considering that the conditions used to prepare the beverages (mix, filter, and package) were mild in comparison to those required to remove the glucose moiety by acid hydrolysis (1 mol L^{-1} HCl, 90 °C). Furthermore, the formation of limonin would likely be concentration dependent, but this relationship does not hold true across all the samples. The second hypothesis seemed equally improbable since the individual lots of LG were recrystallized prior to use. Nevertheless, samples of the LG materials used to prepare the beverages were analyzed for the presence of limonin and through this analysis we determined that the limonin concentrations measured in the beverages were directly attributable to the presence of limonin in the particular lot of LG used to prepare the beverage. Thus limonin was not formed during the preparation or storage of the beverages.

Concurrent with the analysis for limonin, the LG content of the beverages in Groups 1 and 2 was also measured to assess the stability of LG. LG concentration determinations were accomplished utilizing a colorimetric method[5] with some modifications. In brief, forgoing SPE treatment, the samples were diluted 10 or 20:1 with water and acetonitrile to a final acetonitrile concentration of 30% and analyzed directly without further treatment. The resulting colorimetric method was capable of quantitating concentrations of LG as low as 1 mg L^{-1} with a relative uncertainty of <10%. Spike recovery experiments conducted with beverages from Groups 1 and 2 demonstrated that the beverage matrix did not interfere with the method. Analysis of the beverages for their initial LG content indicated that the beverages contained LG at or within 10% of their targeted concentration. The LG content of the beverages was assessed periodically over a 200-day period and found not to vary more than the colorimetric method's uncertainty for all but one sample: the beverage matrix plus 0.25 g LG beverage from Group 2. This particular beverage exhibited a slight but detectable downward trend in its LG concentration (data not shown). However, we were not overly alarmed by this result, because we did not see a corresponding increase in the limonin concentration present in the beverage. The major difference between this beverage and the others was the presence of vitamin B_2.

Beverages in Group 3 were received upon completing the testing of the beverages in Groups 1 and 2. In comparison to the previous batches of beverages, those in Group 3 contained an additional coloring component. Spike recovery experiments confirmed that the beverage matrix did not interfere with the assay. The LG content of the beverage was again confirmed to be within 10% of the targeted concentration of 0.287 g per 254 mL. Samples of the matrix only and matrix plus LG beverages were placed at room temperature on a bench top and under refrigeration in a glass-door refrigerator to simulate conditions found in a retail environment. After 3 days under these conditions the LG content of the beverages had decreased by 42% in the sample stored at room temperature and by 37% for the sample stored in the refrigerator. The concentration of LG in both samples continued to decrease and by the time 3 weeks had passed only one-third of the original concentration remained in the refrigerated sample, and in the room-temperature sample the concentration had diminished to below the detection limit of the assay. The stability study for beverages in Group 3 was terminated after 3 weeks and the beverages in Groups 1 and 2 subjected to the same storage conditions to determine if similar responses would be elicited. Storage under these conditions yielded results analogous to those of Group 3 for the matrix plus LG beverage from Group 2, but not for the beverages in Group 1 or the citrate plus LG beverage in Group 2. We concluded that the presence of vitamin B_2 in the beverages in combination with light exposure during storage led to the loss of LG that was observed.

Following the results from the stability study we faced the dilemma of determining the fate of the disappearing material. Since LG concentrations were monitored by an assay based upon reaction of the furan moiety with DMBA to form an adduct with a chromophore that absorbs at or near 500 nm,[5] the apparent decrease in or absence of LG in the samples could be attributed to precipitation or modification such that the furan ring no longer reacts with the indicator or alternatively forms an adduct that lacks a chromophore captured by the conditions used for the assay. In particular, substitution at the α-position of the furan would prevent the limonoid from reacting with the DMBA reagent.[8,9] Precipitation of LG was ruled out after treatment of the beverages by centrifugation yielded no pellets. UV-visible analysis of samples following treatment with the conditions used for the colorimetric assay revealed no additional absorbance maximums. From this point, we turned to LC-MS and NMR analyses to further investigate the fate of the LG.

Beverage samples were treated by SPE and then analyzed by LC-MS as outlined in the Methods section. The SPE treatment was necessary to accommodate the abbreviated LC method employed. LG was detected in negative mode as its $[M-H]^-$ ion by monitoring at 649.3 *m/z*. In each case, the LC-MS analysis concurred with the results from the colorimetric analysis; samples with LG as determined by the colorimetric method were also found to contain LG by LC-MS, whereas those samples with negative results from the colorimetric assay were found devoid, or contained only traces, of LG. In these samples, concurrent with a decrease in LG, we observed an increasing response for an unknown that eluted prior to LG and exhibited a molecular mass of 681 *m/z*. If the observed mass of 681 *m/z* represents the $[M-H]^-$ of the unknown, then the difference between the mass of this compound and that of LG (649 *m/z*) corresponds to 32 mass units and potentially reflects the addition of two oxygen atoms (2 O, 32 amu) or a methoxy group (CH_3O-, 32 amu). Addition of either of these could yield a compound more polar than LG and may account for the reduced retention time observed for the unknown. Considering the structure of LG and the lack of reactivity obtained from the colorimetric method, the furan moiety appeared to be the most probable site for any modification.

The furan ring present in the structures of limonoids is one of the distinguishing characteristics of this family of compounds, and its detection through chemical or analytical means has routinely been used to identify limonoids. The ^{1}H-NMR resonances of the furan's α and β protons (7.65 (α), 7.36 (α), and 6.63 (β) ppm in the case of LG in CD_3OD) are of particular diagnostic value, since they are individually well resolved and occur down-field from the other proton signals found in the spectra of limonoids. Thus NMR analysis appeared to us as the most expeditious method to probe whether the furan was still intact, and accordingly we prepared our first set of samples for interrogation by NMR by diluting them 1:1 with D_2O prior to analysis. Unfortunately, direct analysis of the diluted beverages was largely unsuccessful, even with suppression of the water signal, on account of the overwhelming citrate concentrations. SPE treatment of the samples followed by reconstitution in CD_3OD remedied this problem. Using dimethyl 3,4-furan dicarboxylate as an internal standard and a series of LG standards ($R^2 > 0.999$) the percentage recovery of LG from the beverages by SPE ranged from 77% to 80%.

Upon examination of the data obtained for the aromatic regions, we found as expected the furan protons present in those spectra obtained from samples formulated without vitamin B_2 or alternatively contained vitamin B_2 but were protected from light, whereas for those samples formulated with vitamin B_2 and exposed to light the furan signals were greatly diminished or undetectable. Displayed in Fig. 3 as representative examples are the aromatic regions of the ^{1}H spectra obtained for the beverages in Group 2. Included in this group are beverages containing only citrate and LG (Fig. 3A) and those formulated with a matrix that includes vitamin B_2 with (Fig. 3B) and without (Fig. 3C) LG. These beverages had been stored protected from light and only in the matrix + LG beverage (Fig. 3B) was a slight decrease in the LG concentration detected (see above: LG stability discussion). In Fig. 3(A) and (B), the α- and β-furan protons of LG are clearly visible; however, some additional proton signals (δ 5.9–6.4 ppm) are also present in Fig. 3(B). These signals cannot be directly accounted to components in the beverage matrix because the same signals are not found in the spectra of the matrix-only beverage (Fig. 3C) and it is probable that they are associated with a product or group of products formed from the degradation of LG.

To test this hypothesis and the hypothesis that LG possesses limited stability when exposed to light in the presence of vitamin B_2 and that the apparent decrease in LG results from modification of the furan moiety, we exposed these three samples to a high-intensity visible light source for 3 days and then reacquired the spectra. Exposure to light had no effect on the LG content of the sample prepared from the citrate plus LG beverage (Fig. 3D). However, in the case of the beverage matrix plus LG sample (Fig. 3E), the furan signals attributed to LG are no longer present. Simultaneous with their loss, the signals found between δ 5.9 and 6.4 ppm have undergone amplification and are accompanied by the appearance of several additional signals down-field (δ 6.66, 7.33 and 7.40 ppm). None of these new signals were observed in the beverage matrix-only sample (Fig. 3F).

The results described above in the proceeding sections supported by HPLC-MS and these NMR

J Sci Food Agric **88**:2194–2200 (2008)
DOI: 10.1002/jsfa

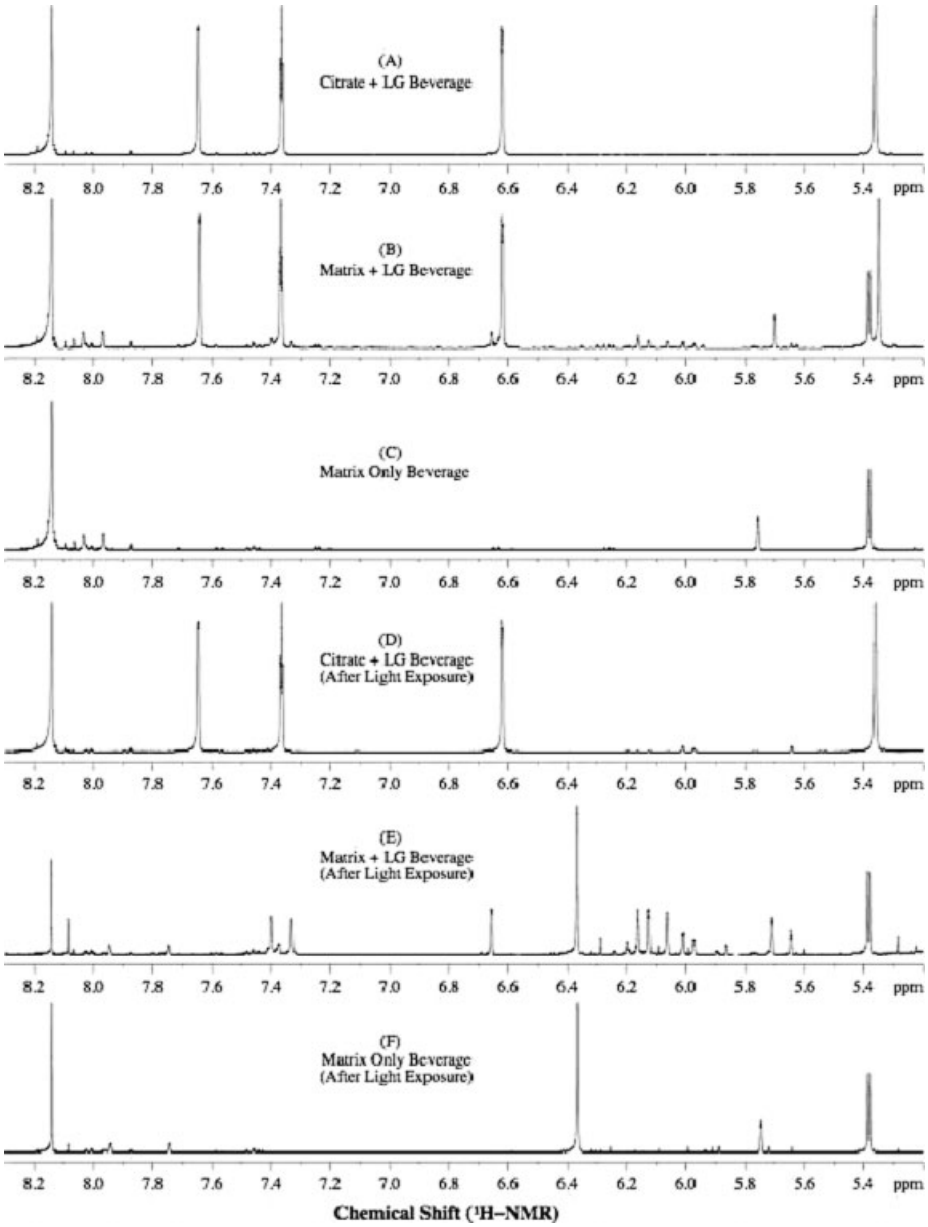


Figure 3. ^{1}H-NMR spectra of beverage extracts pre and post exposure to a high-intensity light source. Beverages were extracted by SPE and reconstituted in CD_3OD. Furan proton signals are found at 7.37 and 7.64 ppm (α) and 6.62 ppm (β).

experiments provide clear objective evidence of the modification of the furan moiety. Based on these same results we propose that the modification is accomplished through the addition of the molecular oxygen across the α-carbons of the furan ring (Fig. 4). The net addition of molecular oxygen to α-unsubstituted furans is not unprecedented, but has been reported to occur through the addition of singlet oxygen, as in the case of the photooxygenation of the *Azadirachta indica* limonoid nimonol.[10] Singlet oxygen[11] can be formed from photo-excited flavins (vitamin B_2) and once formed would reacted with LG without specificity to yield epimers, and this is likely the cause for the appearance of multiple resonances *versus* a single new set of signals in the NMR spectra (Fig. 3D).

The presence of vitamin B_2 in conjunction with exposure to light has been cited as the causative agent in the oxidative fouling of a number of beverages, including beer and milk.[12] Our original intention was to include vitamin B_2 because it is frequently used as a compliance marker in human studies. We did not anticipate B_2 issues because previous studies had demonstrated that *Citrus* limonoids, including LG, were not antioxidants[13,14] (with the exception of those reports with compromised results due to the questionable purity of their materials).[15,16] However, among those studies with compromised results was a report that limonin glucosides possess limited antioxidant activity against superoxide anions.[15] Reflecting upon this observation and the results obtained from our study, some consideration should be given to re-evaluating the reactivity of limonoids against superoxide anions and more potent radical sources using materials of sufficient purity.

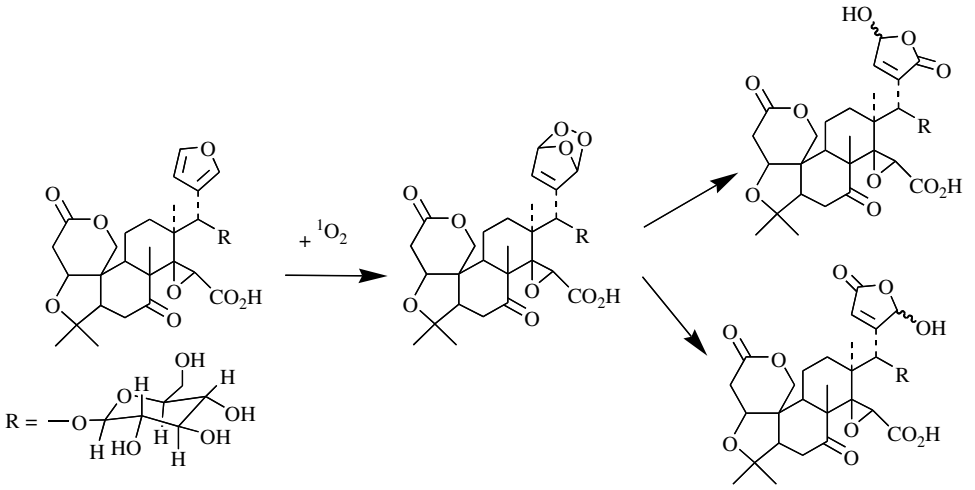


Figure 4. Proposed photooxidative degradation pathway.

CONCLUSION

LG included as a component in a beverage shows good stability with no apparent hydrolysis of the glucose to form the bitter limonoid limonin. Samples that contain riboflavin must be protected from light to prevent the degradation of LG. Although the degradation product has yet to be isolated, LC-MS and NMR data support that the photooxidation of the furan moiety through addition of singlet oxygen is likely the degradation pathway. These results provide a reminder of the potential for components of mixtures to interact and form unexpected and undesired products and that this should not be overlooked when formulating foods and beverages.

ACKNOWLEDGEMENTS

We would like to thank Russell Molyneux (USDA, ARS) and Garry Buettner (University of Iowa) for their helpful and insightful discussions. Thanks to John Beck (USDA, ARS) and Colleen McMahan (USDA, ARS) for their careful reading of the manuscript.

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